



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : A61K 39/395, 37/02, C12Q 1/00 C07K 15/28, 13/00	A1	(11) International Publication Number: WO 91/00742 (43) International Publication Date: 24 January 1991 (24.01.91)
(21) International Application Number: PCT/US90/03556 (22) International Filing Date: 28 June 1990 (28.06.90) (30) Priority data: 376,956 7 July 1989 (07.07.89) US (71) Applicant: E.I. DU PONT DE NEMOURS AND COMPANY [US/SU]; 1007 Market Street, Wilmington, DE 19898 (US). (72) Inventors: GIRI, Judith, Goldstein ; 1148 Putnam Boulevard, Wallingford, PA 19086 (US). HORUK, Richard ; 24 Shadow Oak Court, Mount Laurel, NJ 08054 (US). (74) Agents: HORN, Margaret, A. et al.; E.I. du Pont de Nemours and Company, Legal Department, 1007 Market Street, Wilmington, DE 19898 (US).		(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent)*, DK (European patent), ES (European patent), FR (European patent), GB (European patent), IT (European patent), JP, KR, LU (European patent), NL (European patent), SE (European patent). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: SOLUBLE HUMAN B-CELL INTERLEUKIN-1 RECEPTOR (57) Abstract A soluble, membrane-free and detergent-free interleukin-1 (IL-1) receptor isolated from a human B-cell line is described. The soluble human B-cell IL-1 receptor has an apparent molecular size of about 40 kDa and exhibits at least about a 10-fold greater binding affinity for IL-1 β than IL-1 α .		

DESIGNATIONS OF "DE"

Until further notice, any designation of "DE" in any international application whose international filing date is prior to October 3, 1990, shall have effect in the territory of the Federal Republic of Germany with the exception of the territory of the former German Democratic Republic.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MC	Monaco
AU	Australia	FI	Finland	MG	Madagascar
BB	Barbados	FR	France	ML	Mali
BE	Belgium	GA	Gabon	MR	Mauritania
BF	Burkina Fasso	GB	United Kingdom	MW	Malawi
BG	Bulgaria	GR	Greece	NL	Netherlands
BJ	Benin	HU	Hungary	NO	Norway
BR	Brazil	IT	Italy	PL	Poland
CA	Canada	JP	Japan	RO	Romania
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo			SE	Sweden
CH	Switzerland	KR	Republic of Korea	SN	Senegal
CM	Cameroon	LI	Liechtenstein	SU	Soviet Union
DE	Germany	LK	Sri Lanka	TD	Chad
DK	Denmark	LU	Luxembourg	TG	Togo
				US	United States of America

TitleSoluble Human B-cell Interleukin-1 ReceptorBackground of the Invention

5

Field of the Invention

This invention relates to a soluble interleukin-1 (IL-1) binding protein produced by human B-cells.

10

Background Information

IL-1 is a cytokine, primarily produced by monocytes, that displays a wide spectrum of biological activities in a diverse number of cell types. There are two major species of IL-1, designated IL-1 α and IL-1 β , which share only distant homology in amino acid (aa) sequence, but appear to share a common receptor in many cell types (Kilian, P.L., Kaffka, K.L., Stern, A.S., Woehle, D., Benjamin, W.R., Dechiara, T.M., Gubler, U., Farrar, J.J., Mizel, S.B. and Lomedico, P.T. (1986) J. Immunol., 136, 4509-4514; Dower, S.K., Call, S.M., Gillis, S. and Urdal, D.L. (1986) Proc. Natl. Acad. Sci. USA, 83, 1060-1064; Dower, S.K., Kronheim, S.R., Hopp, T.P., Cantrell, M., Deeley, M., Gillis, S., Henney, C.S. and Urdal, D.L. (1986) Nature, 324, 266-268). IL-1 has been implicated in many disease states including, but not limited, to chronic inflammatory diseases of the joints, lungs, skin, and also chronic inflammatory bowel disease and possibly atherosclerosis (Dannis, V.A. et al. J. of Rheumatology (1987) 14, 33-39; N. Miyasaka et al. (1988), Arthritis and Rheumatism, 31, 480-486; H.-J. Ristow (1987) PNAS, 84, 1940-1944); Satsangi, J. et al. (1987) Clin. Exp. Immunol. 67, 594-605; Dinarello, C.A. (1987) Bull. Inst. Pasteur, 85, 267-285; Alcocer-Vanella, J. et al. (1985) Clin. Exp. Immunol., 59, 666-672).

35

IL-1 plays a central role in the inflammatory and immune responses (See generally: Gery, I., Gershon, R.K. and Waksman, B.H. (1972) J. Exp. Med., 136, 128-142; Dinarello, C.A. (1984) Rev. Infect. Dis. 6, 51-95; Durum, S.K., Schmidt, J.A. and Oppenheim, J.J. (1985) Ann. Rev. Immunol., 3, 263-287). IL-1 activities, which are normally beneficial to the host in defense against infection and tissue injury, can cause severe tissue damage if they persist in chronic diseases.

A direct role for IL-1 in joint destruction in rheumatoid arthritis is suggested by the effect of IL-1 on cartilage breakdown and bone resorption. Synovial fluid and membranes from rheumatoid arthritis patients have elevated IL-1 levels and increased IL-1 production has been associated with other inflammatory diseases such as psoriasis, Crohn's disease, Lyme arthritis (Dannis, V.A. et al. J. of Rheumatology (1987) 14, 33-39; N. Miyasaka et al. (1988), Arthritis and Rheumatism, 31, 480-486; H.-J. Ristow (1987) PNAS, 84, 1940-1944); Satsangi, J. et al. (1987) Clin. Exp. Immunol. 67, 594-605; Dinarello, C.A. (1987) Bull. Inst. Pasteur, 85, 267-285; Alcocer-Vanella, J. et al. (1985) Clin. Exp. Immunol., 59, 666-672; Habicht, G. et al. (1985) J. Immunol. 134, 3147-3154).

In these disease states discussed above, it is presumed that high levels of IL-1 contribute to the progression of the disease. Therefore, one approach to preventing the damaging effects of elevated IL-1 levels found in these disease states is to interfere with binding of IL-1 to its receptor on the surface of responding cells by administering the soluble IL-1-binding protein or shed IL-1 receptor of the present invention. Additionally an assay for screening IL-1 binding would be useful in diagnosing these disease states.

In some disease states, such as cancer and systemic lupus, in which reduced levels of IL-1 appear to result in lower immunoreactivity, exogenous IL-1 can be administered to the patient. In this situation an assay for screening IL-1 binding can be used to monitor the presence of soluble IL-1 receptors or other possible inhibitors of IL-1 which would antagonize the beneficial effects of the exogenously administered IL-1.

In a disease such as acquired immune deficiency syndrome (AIDS) in which it is believed that the presence of an IL-1 inhibitor, rather than reduced IL-1 levels, results in reduced IL-1 activity, an assay for monitoring production of soluble IL-1 receptors can be used prior to administering the exogenous IL-1 (Enk, C. et al. (1986) Scand. J. Immunol. 23, 491-497; Chu et al. 1984, PNAS 81, 4945).

Given the number of disease states in which IL-1 has been implicated there is a need not only for an IL-1 binding protein, which can be administered exogeneously to interfere with IL-1 receptor binding, but also there is a need for an assay for screening IL-1 binding and for screening for soluble IL-1 receptors.

It is known in the art that many polypeptide hormone receptors are transmembrane proteins located on the cell surface and that such transmembrane proteins have an extracellular domain and an intracellular domain. The extracellular domain is a ligand binding domain anchored to the cell by a transmembrane span of hydrophobic amino acid residues. By methods known in the art of molecular biology, it has been possible to clone the genes for a number of such receptors, for example the receptors for insulin, interleukin-2 (IL-2), and epidermal growth factor (EGF), and express their

extracellular ligand-binding domain in a soluble, non-membrane anchored form, free of the constraints of the cell membrane surface (Livneh, E., Prywes, R., Kashles, O., Reiss, N., Sasson, I., Mory, Y., Ulrich, A. and Schlessinger, J. (1986) J. Biol. Chem., 261, 12490-12497; Neeper, M.P., Kuo, L.M., Kiefer, M.C. and Robb, R. J. (1987) J. Immunol., 138, 3532-3538; Whittaker, J. and Okamoto, A. (1988) J. Biol. Chem., 263, 3063-3066).

Soluble receptors are also known to be naturally occurring for a variety of hormones; for example, insulin, IL-2, insulin-like growth factor (IGF-II), EGF, platelet-derived growth factor (PDGF) and Fc receptors (Gavin, J.R., Buell, D.N. and Roth, J. (1972) Science, 178, 168-169; Rubin, L.A., Kurman, C.C., Fritz, M.E., Biddison, W.E., Boutin, B., Yarchoan, R. and Nelson, D.L. (1985) J. Immunol., 135, 3172-3177; MacDonald, R.G., Tepper, M.A., Clairmont, K.B., Perregaux, S.B., and Czac M.P. (1989) J. Biol. Chem., 264, 3256-3261; Weber, W., Gill, G. and Spiess, J. (1984) Science, 224, 294-297; Orchansky, P.L., Escobedo, J.A., and Williams, L.T. (1988) J. Biol. Chem., 263, 15159-15165; Selvearaj, P., Rosse, W.F., Silber, R., and Springer, T.A. (1988) Nature, 333, 565-567; Beguin, Y., Huebers, H.A., Josephson, B., and Finch, C.A. (1988) Proc. Natl. Acad. Sci. USA, 85, 637-640). These soluble or truncated receptors appear to have similar binding properties to those of their membrane-bound counterparts (Livneh, E., Prywes, R., Kashles, O., Reiss, N., Sasson, I., Mory, Y., Ulrich, A. and Schlessinger, J. (1986) J. Biol. Chem., 261, 12490-12497; Neeper, M.P., Kuo, L.M., Kiefer, M.C. and Robb, R. J. (1987) J. Immunol., 138, 3532-3538; Whittaker, J. and Okamoto, A. (1988) J. Biol. Chem., 263, 3063-3066). The soluble or truncated receptor or

binding protein of IL-1 is the focus of the present invention.

Recently, the interleukin-1 (IL-1) receptor gene from a murine T-cell line has been molecularly cloned (Sims et al. (1988) Science, 241, 585-589). The T-cell IL-1 receptor exhibits a structure that is characteristic of the immunoglobulin superfamily. The apparent extracellular segment has three repeating Ig-like domains and two beta pleated sheets, possibly connected by disulfide bonds, that identify this superfamily. The T-cell IL-1 receptor contains seven possible N-linked glycosylation sites and carbohydrate appears to contribute approximately 19% of the molecular weight. The molecular size of the glycosylated extracellular ligand binding domain is approximately 50 kDa. A transmembrane span of 21 amino acids (aa) appears to connect the extracellular domain to a possible intracellular domain of 217 aa.

This T-cell IL-1 receptor has marked differences from the B-cell IL-1 receptor of the present invention. For example, the B-cell receptors bind IL-1 β but bind IL-1 α with low affinity, the B-cell IL-1 receptors internalize slowly and have a lower molecular weight than the T-cell IL-1 receptor. Due to these differences the soluble IL-1 binding protein derived from B-cells, of the present invention, is advantageous over the T-cell IL-1 receptor since the B-cell-derived IL-1 receptor is relatively specific for IL-1 β and binds IL-1 α with low affinity.

Summary of Invention

According to the present invention there is provided an interleukin-1 (IL-1) binding protein which is soluble in buffered, detergent-free aqueous solution having a dissociation constant for IL-1 β of at least 10

nM and a dissociation constant for IL-1 α of greater than 100 nM. Further provided is a pharmaceutical composition useful in treating mammals with IL-1 mediated diseases incorporating the binding protein of the present invention, in a substantially pure form. Furthermore, assays for IL-1-mediated diseases are provided.

Brief Description of the Drawing

Figure 1 is a Scatchard analysis (Scatchard, G. (1949) Ann. N.Y. Acad. Sci., 52, 660-672.) of [¹²⁵I]-labeled IL-1 β binding to intact Raji cells (A) and to cell-free supernatants from Raji cells (B) at 4°C. Binding shown represents specific binding.

Detailed Description of the Invention

We have discovered a soluble IL-1 binding protein produced by human B cells. This protein, a soluble form of the IL-1 receptor present on the surface of the cell, potentially plays a regulatory role in IL-1 action and is useful as a marker of immune-related disease processes or as an exogenously administered therapeutic agent to modulate IL-1-mediated immune-related disease processes by binding to and neutralizing IL-1. Thus, the soluble IL-1 receptor of the present invention, when administered in the form of a pharmaceutical composition to a mammal suffering from an IL-1-mediated disease state, acts to inhibit IL-1 binding to its cell surface receptor by binding and inactivating excess IL-1, and thereby provides a therapeutic treatment alternative in such disease states.

IL-1 has a central immunoregulatory role, by stimulating T and B cell activation and production of lymphokines, leading to specific antibodies and

cytotoxic T cells. In addition, IL-1 has an important role in hematopoiesis (Morrisey, P. et al. (1988) J. Immunol. 140, 4204-4210; R. Neta et al. (1987) J. Immunol. 139, 1861-1866). Several studies have demonstrated reduced IL-1 activity in various immunodeficiency states (Chu et al. 1984, PNAS 81, 4945; Komiyama A. et al. (1988), Clin. Exp. Immunol. 73, 500), burns (Kupper, T.S. et al. (1988) Surgery 98, 200; Yokota M. et al. (1987), Clin. Exp. Immunol. 67 335-342), and cancer (Herman, et al. Cancer Immunol. Immunotherapy 16, 182-185). In some cases, as in AIDS (Enk, C. et al. (1986) Scand. J. Immunol. 23, 491-497), the defect is not inability to produce IL-1 but rather in the inability to respond to IL-1. Assuming that a correlation of the level of soluble IL-1 receptors with disease states is demonstrated, assay of soluble IL-1 receptor levels as a clinical marker has a potentially important diagnostic utility. A kit with reagents to detect soluble IL-1 receptors can be developed using antibodies to purified soluble receptors and an enzyme linked immunosorbent assay (ELISA).

It has been demonstrated that IL-1 elicits its biologic effects by binding to specific cell surface receptors. See: Dower, S. K. and Urdal, D. L. (1987) Immunology Today, 8, 46-51. A variety of evidence from direct binding and competition studies with radiolabeled IL-1 α and IL-1 β has suggested that the IL-1 receptor binding site is generally remarkably well conserved among mammalian species and tissue types (Kilian, P.L., Kaffka, K.L., Stern, A.S., Woehle, D., Benjamin, W.R., Dechiara, T.M., Gubler, U., Farrar, J.J., Mizel, S.B. and Lomedico, P.T. (1986) J. Immunol., 136, 4509-4514; Dower, S.K., Call, S.M., Gillis, S. and Urdal, D.L. (1986) Proc. Natl. Acad. Sci. USA, 83, 1060-1064; Dower, S.K., Kronheim, S.R.,

Hopp, T.P., Cantrell, M., Deeley, M., Gillis, S., Henney, C.S. and Urdal, D.L. (1986) Nature, **324**, 266-268). In addition, chemical labeling of IL-1 receptors with radiolabeled IL-1 resulted (after subtraction of the molecular weight of IL-1) in the specific labeling of proteins of molecular weight 80 to 85 kDa in a variety of cell types examined, suggesting conservation of the structure of the protein. However, biochemical characterization and kinetic analysis of the IL-1 receptor in human B-cell lines has revealed that these cells express a separate class of IL-1 receptors with properties quite distinct from those in T-cells and fibroblasts (Matsushima, K., Akahoshi, T., Furutani, Y. and Oppenheim, J.J. (1986) J. Immunol., **136**, 4496-4502; Chin, J., Limjuco, G., Cameron, P., Sigal, N. and Schmidt, J.A. (1987) Fed. Proc., **46**, 768; Horuk, R., Huang, J.J., Covington, M. and Newton, R.C. (1987) J. Biol. Chem., **262**: 16275-16278). The B-cell receptors bind IL-1 β , but bind IL-1 α with relatively low affinity (with binding dissociation constant >50 nM), or not at all. Moreover, the B-cell IL-1 receptor internalizes slowly compared to T-cell IL-1 receptors (Horuk, R., Huang, J.J., Covington, M. and Newton, R.C. (1987) J. Biol. Chem., **262**: 16275-16278).

We have determined that there are also marked differences in the relative binding affinities of certain IL-1 β analogs suggesting that there are structural differences in the way IL-1 α and IL-1 β are recognized by receptors on these two cell types (Horuk, R., Huang, J.J., Covington, M. and Newton, R.C. (1987) J. Biol. Chem., **262**: 16275-16278). In addition, chemical crosslinking studies have shown that the IL-1 receptor on B-cells has a lower molecular weight than that on T-cells (68 kDa compared to 80 kDa) (Horuk, R.,

Huang, J.J., Covington, M. and Newton, R.C. (1987) J. Biol. Chem., 262: 16275-16278).

Several inhibitors of IL-1 activity have been described. The best characterized is a protein known as uromodulin or Tamm-Horsfall protein (Pennica, D., et al. (1987) Science 236, 83-88; Hession, C. et al. (1987) Science 23, 1479-1484). Unlike the soluble IL-1 receptor described herein, uromodulin is produced in the kidney only, has a larger size (85 kDa compared to 40 to 50 kDa) and, unlike the soluble IL-1 receptor of this invention, binds both IL-1 and TNF. An inhibitor of IL-1 produced by Epstein Barr Virus (EBV)-infected B-cells has also been described by Scala et al. (Scala, et al. (1984) JEM 159, 1637-1652) with a 95 kDa molecular weight, and which appears to be a distinct factor capable of inhibiting IL-1 function in T-cells.

The following experiments and the data collected therefrom are meant to further illustrate the properties of the soluble IL-1 binding protein of the present invention and also to further demonstrate its utility.

Materials

Human recombinant IL-1 β expressed in E. coli was purified to homogeneity according to methods known in the art (Huang, J.J., Newton, R.C., Pezzella, K., Covington, M., Tamblyn, T., Rutledge, S.J., Kelley, M. and Lin, Y. (1987) Mol. Biol. Med., 4, 169-181). ¹²⁵I-labeled IL-1, [¹²⁵I]IL-1 (E. I. du Pont de Nemours and Company/NEN Products, Boston, MA) (specific activity 143-180 μ Ci per μ g), labeled using the Bolton Hunter reagent was used. Reagents for electrophoresis were from Bio-Rad (Richmond, CA). All other reagent grade chemicals were from Sigma (St. Louis, MO).

Cell Culture

Raji human B-lymphoma cells were obtained from the American Type Culture Collection Rockville, MD (ATCC CCL 86) and were maintained in RPMI 1640 medium containing 10% fetal calf serum or in serum-free medium. The cells were passaged weekly and the medium was changed two additional times weekly. Cell viability was assessed by trypan blue exclusion and cell number was determined by counting the cells in a hemacytometer.

Data Analysis

Equilibrium binding data were analyzed using the nonlinear regression program LIGAND as adapted for the IBM PC by McPherson (See: McPherson, G.A. (1983) Comp. Prog. in Biomed., 17, 107-111) from Elsevier-BIOSOFT.

Receptor Binding Assay of ^{125}I -labeled IL-1 to Intact Cells

Raji cells (1×10^7 cells per mL) were incubated with ^{125}I -labeled IL-1 (0.2 to 1 nM) and varying concentrations of unlabeled IL-1 at the appropriate temperature for the designated times. The incubation was terminated by removing aliquots from the cell suspension and separating cells from buffer by centrifugation through a silicon/paraffin oil mixture, as known in the art. The present assay was adapted from an assay previously reported for the detection of IL-2 receptors. See: Neeper, M. P., Kuo, L. M., Kiefer, M.C. and Robb, R. J. (1987) J. Immunol., 138, 3532-3538. Non-specific binding was determined in the presence of $1 \mu\text{M}$ unlabeled IL-1. The biologic specific activity of the radiolabeled IL-1 was the same as that of unlabeled IL-1.

Preparation of Soluble IL-1 Receptor and Quantification
by IL-1 Binding Assay

Crude culture supernatants were obtained from Raji cells seeded at a cellular density of 3 to 6×10^5 cells per mL and grown in culture over a period of four days. Culture medium from cells grown both in the presence and absence of serum was collected by centrifugation at $1000 \times g$ for 5 min, the supernatants were poured off, rapidly chilled to 4°C and concentrated 10-fold by ultrafiltration (Amicon DC10 membrane with a 10 kDa molecular weight cut-off) in the presence of the protease inhibitors leupeptin and aprotinin (each at $10 \mu\text{g}$ per mL). The concentrated supernatant samples were centrifuged at $100,000 \times g$ for 90 min and the supernatants gently aspirated off. Four mL of 0.1% bovine gamma globulin were added to 100 mLs of the clarified supernatant followed by 100 mL of ice cold 20% polyethylene glycol 8000 in phosphate buffered saline (PBS), pH 7.6, containing 0.2 mM phenylmethylsulfonyl fluoride (PMSF) and $5 \mu\text{g}$ per mL of leupeptin and aprotinin. After standing for about 5 min at 4°C the cloudy supernatants were centrifuged at $48,000 \times g$ for 30 min. The supernatants after centrifugation were discarded and the precipitated protein pellets were washed twice with PBS and then resuspended in PBS, pH 7.6, containing 0.2 mM PMSF and $10 \mu\text{g}$ per mL of leupeptin and aprotinin. The resuspended protein pellets were then assayed for IL-1 binding, as described by Marshall et al. (1985) J. Biol. Chem., 260, 4128-4135 for the insulin receptor. Briefly, $100 \mu\text{L}$ aliquots of precipitated proteins were incubated with $15 \mu\text{L}$ of ^{125}I -labeled IL-1 in a total volume of $150 \mu\text{L}$ at 4°C for 16 to 24 h. Binding was carried out using a range of radiolabeled IL-1 concentrations in the presence and absence of $1 \mu\text{M}$

unlabeled IL-1. Free ^{125}I -labeled IL-1 was separated from receptor bound ligand by adding 150 μL of 20% polyethylene glycol and centrifuging the mixture at 11,000x g for 5 min. The pellet was washed twice with PBS (500 μL), and the tip of the microcentrifuge tube was cut off and the radioactivity counted. Nonspecific binding, defined as the amount of ^{125}I -labeled IL-1 remaining in the pellet in the presence of excess unlabeled IL-1 (1 μM), was less than 30% of total binding and could be reduced to 10% by resuspension of the pellet followed by reprecipitation with 10% polyethylene glycol, as described above. IL-1 binding data for cell supernates are expressed in terms of cell equivalents. For example, 1×10^7 cell equivalents refers to the supernates from 1×10^7 cells. Protein was determined using the micro BCA protein assay reagent (Pierce, Rockford, IL).

Using the above described assay for detecting soluble, non-membrane-bound IL-1 receptors, the following experiments were conducted with human B cell Raji cell line. An IL-1 receptor-negative Raji subclone, designated Raji subclone 0.7, was the control. The data from several binding experiments are shown in Table 1. With the IL-1 receptor-positive Raji cells (subclone 22/23), IL-1 β exhibited high level binding to cell surface receptors (approximately 38,000 counts per minute (cpm) of radiolabeled IL-1 β bound per 10^7 cell equivalents; Table 1), whereas only low levels (around 700 cpm per 10^7 cell equivalents; Table 1) of IL-1 β bound to the receptor-negative Raji cell line (subclone 0.7). In addition, significant levels of IL-1 binding protein (around 16,000 cpm of radiolabeled IL-1 bound per 10^7 cell equivalents; Table 1) were detected in the culture supernatants of IL-1 receptor-positive cells, compared to no detectable IL-1 binding protein in the supernatants from the receptor-negative cells.

TABLE 1

5 IL-1 β binding to receptors on intact cells and cell-free culture supernatants from a Raji IL-1 receptor-positive subclone (22/23) and an IL-1 receptor-negative Raji subclone (0.7). Data are the means from three experiments \pm SEM. Binding data for supernatants are expressed in terms of cell equivalents.

10

<u>Cell Line</u>	<u>Specific ^{125}I-labeled IL-1β binding (cpm per 10^7 cells)</u>	
	<u>Intact cells</u>	<u>Supernatants</u>
15 Raji clone 22/23	38,868 \pm 5,820	15,933 \pm 1,601
Raji clone 0.7	690 \pm 129	0

20

The results indicate that the specific IL-1 β binding detected is due to a soluble form of the IL-1 receptor in view of the following analysis. The cell-free supernatants collected were centrifuged at 100,000x g for 90 min and then passed through a 0.22 μm filter and therefore meet the criteria which establish the material as soluble, since membrane-bound proteins are removed by these procedures. The inability to detect a soluble IL-1 binding factor in the cell-free supernatants from the Raji IL-1 receptor-negative subclone cells excludes the possibility that the IL-1 binding results from interactions between IL-1 and a protein present in the serum-containing medium alone.

25

30

In order to determine the specificity of the IL-1 binding protein, we examined the ability of various ^{125}I -labeled ligands, including IL-1 β , IL-1 α , IL-2, TNF

35

and EGF, to specifically bind to the soluble IL-1 receptor. As shown in Table 2, only IL-1 β and IL-1 α were able to specifically bind the soluble IL-1 receptor from Raji cells (Table 2A). At equivalent
5 IL-1 concentrations (200 to 1000 pM) IL-1 β binding was approximately 16-fold greater than the binding exhibited by IL-1 α . The binding of IL-1 α and IL-1 β to the plasma membrane-bound receptors on Raji cells showed a similar specificity of binding, i.e., IL-1 β
10 binding was 13-fold higher than IL-1 α (Table 2). We have determined by standard Scatchard analysis, that the K_D for IL-1 β for the binding to the IL-1 receptor on intact Raji cells is about 1 nM, while that for IL-1 α is about 15 nM. Thus, the soluble IL-1 binding
15 protein derived from Raji cells is identical to the cell membrane-bound form of the IL-1 receptor in its ability to discriminate between IL-1 β and IL-1 α . The soluble IL-1 receptor was specific for IL-1 and did not bind other ligands such as IL-2, TNF, and EGF (Table
20 2).

TABLE 2

5 Specificity of (A) Raji soluble IL-1 receptor and
 (B) Raji plasma membrane receptor for IL-1 β .
 Polyethylene glycol-precipitated, concentrated shed
 receptors (1×10^7 cell equivalents) (A) and cells ($1 \times$
 10 7 per mL) (B) were incubated with 125 I-labeled IL-1 β ,
 IL-1 α , IL-2, TNF, or EGF (500 pM) in the presence or
 10 absence of unlabeled ligands (500 nM). Receptor
 binding assays were carried out, as described for IL-1.
 The specific radioactivity (cpm/molecule) was
 approximately the same for each of the ligands tested.
 The binding shown here represents specific binding and
 is the mean of three experiments \pm SEM.

15	<u>Ligand</u>	<u>Specific 125I-labeled ligand binding (cpm per 10^7 cells)</u>
	(A)	
	IL-1 β	9395 \pm 1380
	IL-1 α	593 \pm 290
	IL-2	32
	TNF	78
20	EGF	47
	(B)	
	IL-1 β	3500
25	IL-1 α	500

Analysis of the equilibrium binding properties of
 the soluble IL-1 receptor using the method described by
 Scatchard, G. (1949) Ann N.Y. Acad. Sci., 52, 660-672
 30 yielded the data shown in Figure 1. Also shown, for
 comparison, is an IL-1 receptor binding analysis
 carried out with intact cells. Both studies were
 carried out at 4°C. Cells (1×10^7) and PEG
 precipitated concentrated supernatants (100 μ L; 1×10^7
 35 cell equivalents) were incubated with varying

concentrations of radiolabeled IL-1 at 4°C for 20 h. Binding shown represents specific binding. The dissociation constant (K_D) for binding to intact cells was about 0.8 nM compared to about 8 nM for the binding of IL-1 β to the soluble receptor. Thus, the soluble receptor has an approximately 10-fold lower affinity for IL-1 β than the cell membrane-bound IL-1 receptor. This is consistent with what has been found for many other soluble hormone receptors, including insulin, IL-2, and EGF, each of which has a 10-fold or greater reduction in affinity for their respective ligands, compared to the membrane-bound receptor (Gavin, J.R., Buell, D.N. and Roth, J. (1972) Science, 178, 168-169; Rubin, L.A., Kurman, C.C., Fritz, M.E., Biddison, W.E., Boutin, B., Yarchoan, R. and Nelson, D.L. (1985) J. Immunol., 135, 3172-3177; Weber, W., Gill, G. and Spiess, J. (1984) Science, 224, 294-297).

Further evidence that the soluble IL-1 binding protein is a soluble form of the membrane-bound IL-1 receptor comes from antibody inhibition studies shown in Table 3. Antibodies to the IL-1 receptor were raised by injecting several mice with repeated doses of Raji subclone 22/23. A 1:50 dose of antiserum from one of the injected animals inhibited IL-1 receptor binding to intact Raji cells by about 50%. In contrast a 1:50 dose of preimmune serum had no effect on IL-1 binding. In addition to inhibiting binding to intact cells, the antiserum also inhibited binding to the soluble IL-1 receptor to a similar degree. This result provides evidence that the ligand-binding domain of the membrane-bound IL-1 receptor and the soluble IL-1 binding protein have structural features in common, which are immunologically cross-reactive.

TABLE 3

5 Inhibition of IL-1 binding by antibodies to the membrane-bound IL-1 receptor. Intact cells (1×10^7 cells per mL) and polyethylene glycol-precipitated and concentrated shed receptors (1×10^7 cell equivalents) were assayed for ^{125}I -labeled IL-1 β binding, in the presence or absence of antiserum raised against the membrane-bound Raji IL-1 receptor in mice, as described above. Antiserum at a final dilution of 1:50 was used; preimmune serum at a similar concentration was used as a control for non-specific inhibition. Data represent the mean \pm SEM from three experiments.

15	Specific ^{125}I -labeled IL-1 β binding (cpm per 10^7 cells)	
	<u>Conditions</u>	<u>Supernatants</u>
	Intact cells	
	Control (no serum)	37,821 \pm 4,184
	Preimmune Serum	18,989 \pm 2,109
	Receptor Antibodies	33,556 \pm 3,531
		17,490 \pm 3,109
		9,557 \pm 851

20

In addition, treatment of the soluble IL-1 receptor with 300 μg per mL of trypsin for 30 min at 37°C reduced the IL-1 binding activity of the soluble receptor by almost 91%; specific [^{125}I]IL-1 binding to untreated soluble receptor was 7026 cpm compared to 640 cpm to trypsin-treated soluble receptor. These data confirm that the soluble IL-1 binding factor is in fact a protein.

25

30

Preparation of IL-1 Affinity Column and Affinity Purification of Shed Receptor from Raji Cell Supernatants

35

Recombinant IL-1 β was coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia, Piscataway, NJ), according to the manufacturers suggestions, to yield

IL-1-Sepharose 4B. Under these conditions the gel had a coupling capacity of 3-5 mg of IL-1 β per mL of gel. Crude culture supernatants from Raji cells (0.4 to 1.0 x 10⁹ cell equivalents) were concentrated, centrifuged at 100,000x g for 90 min and the supernatants gently aspirated off. The concentrated supernatants were incubated with IL-1-Sepharose 4B overnight at 4°C in an end-over-end rotator. After incubation the gel suspension was centrifuged, 5000x g for 1 min, supernatant was removed and the gel was resuspended in 2 mL of PBS. The resuspended gel was poured into a column and washed with two bed volumes of PBS. The bound receptors were eluted with 2 mL of 0.1 M glycine, pH 3.0, and the pH of the eluate was adjusted to 7.6 with 1 M Trizma base. The presence of receptor in the eluted fractions was detected by the soluble IL-1 receptor binding assay, described above.

Consistent with the above data, when cell-free extracts of Raji clone 22/23 were passed over an IL-1 β affinity column, considerable amounts of IL-1 binding activity were specifically bound to the column (Table 4).

As shown in Table 4, the soluble IL-1 receptor was purified about 400-fold using the IL-1 β -Sepharose affinity chromatography column step. The soluble IL-1 receptor may be purified to homogeneity by one skilled in the art of protein purification, using standard protein purification procedures. Standard protein purification materials and methods include those such as supplied by Pharmacia LKB Biotechnology Products (Piscataway, NJ).

TABLE 4

Purification of soluble IL-1 β receptors. Raji cell culture supernatants from 1.8×10^9 Raji cells were analyzed by IL-1 β -Sepharose affinity chromatography.

<u>Sample</u>	<u>IL-1 Binding Activity</u>	
	<u>cpm $\times 10^3$/mL</u>	<u>cpm/μg protein</u>
Total loaded	1.47	7.35
Flow-through (Unbound)	0.76	3.45
Acid eluate	106.00	2944 (= 400-fold purification)

Raji cells were also solubilized in 1% Triton X-100 at 4°C for 30 min. The solubilized cells were centrifuged at $100,000 \times g$ for 90 min and the supernatants were purified by affinity chromatography. Affinity purification of the detergent-solubilized Raji IL-1 receptors was carried out as described above except that 0.1% Triton X-100 was present in the buffers during all stages of the purification and in the receptor binding assay, described above.

Superose 12 Gel Permeation Column Chromatography of Affinity-purified Soluble IL-1 Receptor

An estimate of the molecular size of the soluble IL-1 binding protein was provided from studies in which the affinity purified Triton solubilized plasma membrane IL-1 receptor and the soluble IL-1 binding protein were subjected to gel permeation chromatography on a Superose 12 (Pharmacia, Piscataway, NJ) column.

Affinity-purified shed IL-1 receptors, prepared as described above, were chromatographed on a Superose 12

column (1 x 30 cm) equilibrated with PBS, pH 7.6. The eluted fractions were assayed for ^{125}I -labeled IL-1 binding activity, as described above.

Affinity-purified detergent-solubilized IL-1 receptors from Raji cells were also purified by FPLC under the conditions described above, except that 0.1% Triton X-100 was present during the chromatography. Bovine serum albumin and ovalbumin were used to calibrate the column.

Gel permeation chromatography of the Triton solubilized cell receptor yielded an IL-1 binding peak with a molecular weight of approximately 68 kDa. This is consistent with our previous observation that the crosslinked Raji membrane-bound IL-1 receptor analyzed by SDS-PAGE has a molecular weight of about 68 kDa (Horuk, R., Huang, J.J., Covington, M. and Newton, R.C. (1987) J. Biol. Chem., 262: 16275-16278). Gel chromatography of the soluble IL-1 binding protein yielded an IL-1 binding peak with a molecular weight of approximately 40 kDa. This is much smaller than the molecular weight reported for other IL-1 binding proteins previously described (Pennica, D., et al. (1987) Science 236, 83-88; Hession, C. et al. (1987) Science 23, 1479-1484; Scala, et al. (1984) JEM 159, 1637-1652). The best characterized of these is uromodulin (Pennica, D., et al. (1987) Science 236, 83-88; Hession, C. et al. (1987) Science 23, 1479-1484). Unlike the soluble IL-1 binding protein described herein, uromodulin is only produced in kidneys, has a larger size (85 kDa), and binds both IL-1 and TNF. An inhibitor of IL-1 produced by EBV-transformed B-cells has also been described (Scala, et al. (1984) JEM 159, 1637-1652). This protein has a molecular weight of 95 kDa and appears to inhibit IL-1 function in T-cells.

Inhibition of IL-1 β Binding to Membrane-bound
IL-1 Receptor by Soluble IL-1 β Receptor

5 The inhibition of ^{125}I -labeled IL-1 binding to Raji
cells by increasing concentrations of soluble IL-1
binding protein is shown in Table 5. As can be seen,
the soluble IL-1 binding protein competes with intact
cell membrane-associated IL-1 receptors for IL-1
binding in a dose-responsive manner.

10 Raji cells (1×10^7 cells per mL) were incubated
with radiolabeled IL-1 β (250 pM) and with increasing
concentrations of affinity purified IL-1 binding
protein. Incubations were at 37°C for 3 h and binding
was terminated as described above. Specific binding in
15 the absence of soluble binding protein was expressed as
100% and the percentage of initial specific binding in
the presence of increasing concentrations of soluble
binding protein was calculated.

20

25

30

35

TABLE 5

Inhibition of ^{125}I -IL-1 binding to Raji cells by increasing concentrations of soluble IL-1 binding protein.

	Concentration of Soluble Receptor (μL Added)	% of Initial Binding
	0	100
15	2	93
	5	92
	10	82
	20	68
	50	50
20	100	23
	200	7

Inhibition of IL-1-Induced Prostaglandin
E2 Production by Soluble IL-1 Receptor

In order to examine whether soluble IL-1 receptor protein would inhibit IL-1-mediated biological activity, we tested whether soluble IL-1 receptor inhibited IL-1-induced prostaglandin E2 production.

The Gin-1 human fibroblast cell line, obtained from the ATCC, was plated in 96 well plates at 2×10^5 cells/mL and 100 μL /well in DMEM with 10% FBS. After overnight incubation, the cells were washed once with medium and 100 μL of medium readdded. Samples of Raji

soluble IL-1 binding protein in medium were added in 50 μ L volumes to the appropriate wells with medium added to control wells. The control wells were assayed in quadruplicate while the binding protein added wells were assayed in duplicate. The soluble IL-1 binding protein had been previously purified by IL-1 affinity chromatography. Human recombinant IL-1 β , diluted in medium, was added to wells to give a final concentration of 1 ng/mL. Samples of supernate were collected after 2, 4, 6, and 8 hours and assayed for prostaglandin E2 level by radioimmunoassay (DuPont-NEN, Boston, MA).

The results of the time course for PGE2 production are shown in Table 6. Values are the average \pm standard deviation. After 8 hours, there is an 83% inhibition of PGE2 production by added soluble IL-1 receptor protein.

TABLE 6

Inhibition of IL-1-mediated PGE2
by Soluble IL-1 Receptor

Time Following IL-1 β	PGE2 (pg/mL)	
	No Added Soluble Receptor	50 μ L Added Soluble Receptor
2	224 \pm 126	170 \pm 15
4	171 \pm 265	670 \pm 96
6	1939 \pm 454	188 \pm 10
8	2336 \pm 330	505 \pm 48

Dosage and Administration

Purified soluble IL-1 receptor can be administered as a sterile, nonpyrogenic parenteral solution. The aqueous parenteral vehicle could be, for example, Sterile Water for Injection USP, 0.9% Sodium Chloride for Injection USP or 5% Dextrose Injection USP. Pharmaceutical compositions of the present invention comprise an effective amount of purified soluble IL-1 receptor and a pharmaceutically acceptable carrier such as listed above, and optionally additional excipients such as preservatives and buffers as known to those skilled in the art of pharmaceutical formulations. See, for example, Remington's Pharmaceutical Sciences, a standard reference in the field. Soluble IL-1 receptor may be prepared in a stable formulation ready for administration, or for dilution in an appropriate intravenous solution. Preferably, to increase product shelf life, the soluble IL-1 receptor may be formulated, e.g., as a sterile lyophilized powder to be reconstituted aseptically or as a buffered solution.

As is known to those skilled in the art, individual patient dosage will vary depending upon known factors such as the pharmacodynamic characteristics of the particular active ingredient, and its mode and the route of administration; the age, health, and weight of the recipient; the nature and extent of symptoms; kind of concurrent treatment, frequency of treatment, and the effect desired.

Immunological Assays for Detecting and Measuring Soluble IL-1 Receptors in a Biological Fluid

Standard RIA or ELISA procedures may also be used to detect and measure soluble IL-1 receptor proteins in a biological fluid. For example, a heterogenous sandwich ELISA method may be used, in which the antigen

captured antibody specific for soluble IL-1 receptor is immobilized on a solid surface and used to bind soluble IL-1 receptor present in a biological fluid which is contacted with the immobilized capture antibody. A second IL-1 receptor-specific antibody, which is labeled with a reporter, is used as a detection antibody. The detection antibody binds specifically to the captured antigen to form an antibody-antigen-antibody sandwich, with the reporter signal being a function of the amount of antigen (soluble IL-1 receptor) in the biological sample. Those skilled in the art will appreciate that the detector system can include any reagent or combination of reagents suitable for detecting proteins in an ELISA format. Examples of reporters include enzymes, such as horseradish peroxidase and alkaline phosphatase, radioisotopes, and chemiluminescent, fluorogenic, or electrochemical materials. Various coupling techniques are known to those skilled in the art. Where the reporter is an enzyme, the enzyme in the sandwich acts on a substrate solution to produce color in the solution, and the absorbance of the solution is measured and correlated to the presence or quantity of antigen in the sample.

The soluble IL-1 receptor-specific capture and detection antibodies may be polyclonal antibodies or monoclonal antibodies, and may be antibody produced by the same animal or hybridoma, or antibodies produced by different animals or hybridomas.

Polyclonal antibodies specific for the soluble IL-1 receptor protein are prepared using standard procedures. Briefly, an animal is immunized using the purified soluble IL-1 receptor as an immunogen to elicit an humoral immune response in the animal. Following immunization, blood is removed from the animal, serum is obtained from the coagulated blood,

and soluble IL-1 receptor-specific antibodies in the serum may be used directly or purified from the serum.

Monoclonal antibodies specific to the soluble IL-1 receptor can be generated using the purified soluble IL-1 receptor as immunogen. Kohler and Milstein, Nature, 256, 495 (1975), first described methods of making monoclonal antibodies by fusing spleen cells from an immunized mouse to a drug-resistant plasmacytoma cell line and isolating the hybrid clones by growth on selective medium. Monoclonal antibodies can overcome many of the problems associated with the use of polyclonal antisera; namely purity, specificity, homogeneity and availability.

Monoclonal antibodies can be prepared by methods known to one skilled in the art, such as, by the method set forth herein. Briefly, an animal is immunized with purified soluble IL-1 receptor protein, an antibody-producing organ is subsequently harvested, a cellular homogenate is prepared, and cells in the homogenate are fused with tumor cells to produce hybridoma cells. Hybridoma cells which produce antibody specific for soluble IL-1 receptor are selected by assaying for binding of hybridoma-produced antibody to soluble IL-1 receptor using standard radio-labeled soluble IL-1 receptor and radio-immunoassay (RIA) procedures, or soluble IL-1 receptor immobilized on a solid support and standard enzyme-linked immunoassay (ELISA) procedures. Hybridomas producing monoclonal antibody specific for soluble IL-1 receptor may be expanded and monoclonal antibody produced may be harvested.

Typically, an animal is immunized with the antigen of interest emulsified in an adjuvant and boosted at regular intervals. The serum is assayed for the presence of the desired antibody by any convenient method, frequently an ELISA or a RIA. When an

acceptable titer of antibody is detectable in the serum, the animal is sacrificed and the spleen is removed aseptically for fusion.

5 Several different murine (mouse) myeloma cell lines deficient in hypoxanthine guanine phosphoribosyl transferase (HGPRT) are known to be suitable as fusion partners. The features of some of these cell lines are described in Current Topics in Microbiology and Immunology, 81, F. Melchers, M. Potter, and N. Warner,
10 ed., Springer-Verlag, 1978.

Fusion is carried out most commonly by using polyethylene glycol as a fusion promoter. After fusion, the cells are diluted, and cultured in a selective medium containing hypoxanthine, aminopterin
15 and thymidine (HAT). Cells may be supplemented with insulin to enhance the formation and growth of hybridomas. When sufficient cell growth has occurred, the culture supernatant is sampled and tested by any convenient means, frequently ELISA or RIA. Those
20 cultures which contain antibody of interest are then cloned by limiting dilution, re-tested and expanded.

Large volumes of antibody can then be obtained by growing the hybridoma in vitro and harvesting the culture supernatant. Antibodies may also be harvested
25 from the ascites fluid of syngeneic mice which have been injected intraperitoneally with the hybridoma cells.

The antibody is purified using techniques well-known in the art. Chromatography on staphylococcal
30 protein A is one such method. The monoclonal antibody thus generated can be characterized by its immunoglobulin class and subclass.

35

CLAIMS

WHAT IS CLAIMED IS:

1. An interleukin-1 (IL-1) binding protein, soluble
in buffered detergent-free aqueous solution,
having a dissociation constant for IL-1 β of at
least about 10 nM and a dissociation constant for
IL-1 α of greater than 100 nM.
2. An interleukin-1 (IL-1) binding protein of Claim 1
which is substantially pure.
3. An interleukin-1 (IL-1) binding protein of Claim 1
derived from a human B-cell.
4. A binding protein of Claim 3 which is
substantially pure.
5. A pharmaceutical composition comprising a
pharmaceutically acceptable carrier and an
effective amount of a protein of Claim 2.
6. A pharmaceutical composition comprising a
pharmaceutically acceptable carrier and an
effective amount of a protein of Claim 4.
7. A method of treating an IL-1-mediated disease in a
mammal comprising administering to the mammal an
amount of a composition of Claim 5 sufficient to
bind and neutralize the biological activity of
endogenous IL-1 β
8. A method of treating an IL-1-mediated disease in a
mammal comprising administering to the mammal an
amount of a composition of Claim 6 sufficient to

bind and neutralize the biological activity of endogenous IL-1 β .

- 5
9. A method to assist in the diagnosis of IL-1-mediated disease comprising detecting soluble IL-1 receptor in a biological sample by contacting the biological sample with an antibody specific for an IL-1 binding protein of Claim 1, and measuring immunoreactivity.
- 10
10. A method to assist in the diagnosis of IL-1-mediated disease comprising detecting soluble IL-1 receptors in a biological sample by contacting the biological sample with an antibody specific for an IL-1 binding protein of Claim 3, and detecting immunoreactivity.
- 15
11. A method of Claim 9 wherein the antibody is a monoclonal antibody.
- 20
12. A method of Claim 10 wherein the antibody is a monoclonal antibody.
- 25
13. A method of Claim 9 wherein an enzyme-linked immunoassay is used for measuring the level of soluble IL-1 receptor in the biological sample.
- 30
14. A method of Claim 10 wherein an enzyme-linked immunoassay is used for measuring the level of soluble IL-1 receptor in the biological sample.
- 35

1/1

FIG. 1A

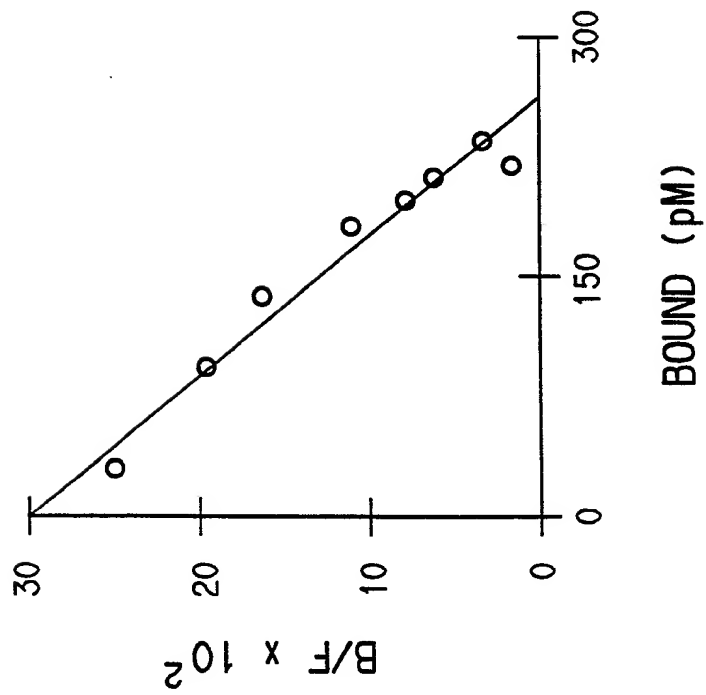
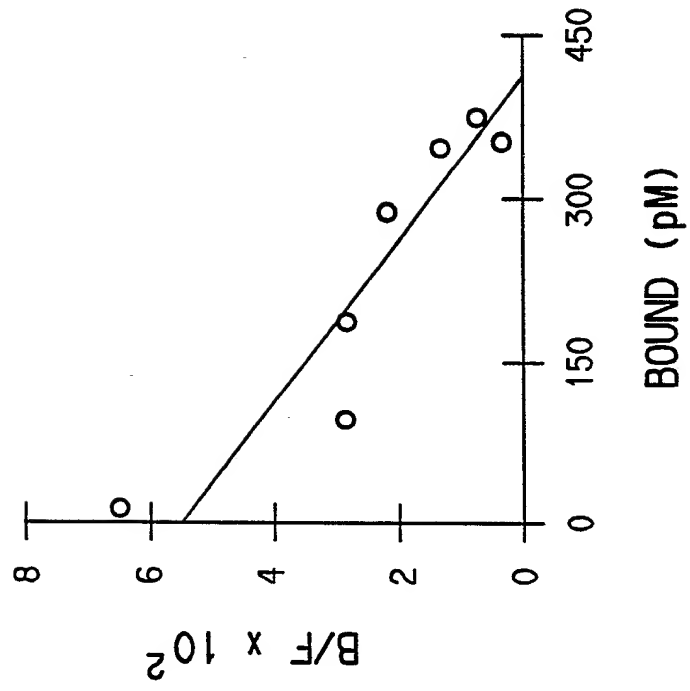


FIG. 1B



INTERNATIONAL SEARCH REPORT

International Application No

PCT/U390/03556

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ³

According to International Patent Classification (IPC) or to both National Classification and IPC
 IPC(5):A61K 39/395, 37/02; C12Q 1/00; C07K 15/28, 13/00
 U.S. CL.: 530/350, 387; 514/12; 435/7

II. FIELDS SEARCHED

Minimum Documentation Searched ⁴

Classification System	Classification Symbols
U.S.	530/350, 387 514/12, 435/7

Documentation Searched other than Minimum Documentation
 to the Extent that such Documents are Included in the Fields Searched ⁶

Automated Patent Search, Chemical Abstract Service

III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴

Category [*]	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
A	Lymphokine Research, vol. 8, no. 3, March 1989, J. Bertoglio, "Interleukin 1 and Interleukin 1 Receptors in Human B-Cell Lines", pages 301-303. See entire document.	1-14
X, P	Proc. Natl. Acad. Sci., USA, vol. 86, October 1989, Chizzonite et al., "Two high-affinity interleukin 1 receptors represent separate gene products", pages 8029-8033. See abstract.	1-8
X, P	Proc. Natl. Acad. Sci., USA, vol. 86, October 1989, Bomsztyk et al. "Evidence for different interleukin 1 receptors in murine B- and T-cell lines." pages 8034-8038. See abstract.	1-8

^{*} Special categories of cited documents: ¹⁵

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search ²

20 September 1990

Date of Mailing of this International Search Report ¹

20 NOV 1990

International Searching Authority ¹

ISA/US

Signature of Authorized Officer ¹⁰

NINA OSSANNA, Ph.D.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No ¹⁸
------------	----------------------------------------------------------------------------------------------------------------	------------------------------------

X	The Journal of Immunology, vol. 136, no. 12, 15 June 1986, Matsushima, et al., "Properties of a specific interleukin 1 (IL 1) receptor on human Epstein Barr virus-transformed B lymphocytes: identity of the receptor for IL 1 α and IL 1 β ". pp. 4496-4502. See abstract.	1-6
X, P	US, A, 4,935,343 (Allison et al.) 19 June 1990, See claims 1-11 and column 7, lines 12-27.	1-14